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THE RESPIRATION OF THE PROTOZOAN PARASITE, *EIMERIA TENELLA*[1]

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INTRODUCTION

The economic importance of the protozoan parasite, *Eimeria tenella,* has stimulated a great deal of research along the lines of pathology, specificity, prevention, and its resistance to environmental factors such as temperature and chemicals. Certain of the more academic studies of this parasite have been somewhat neglected. The authors are unaware of research which measured the metabolic rate of the parasite itself or of the tissues invaded by the parasite. It was to supplement our knowledge along this line that work on the metabolic rate of the parasite and invaded tissues was undertaken.

MATERIALS AND METHODS

Source of the oöcysts and infected tissues.—Single Comb White Leghorn chickens supplied by the Department of Poultry Husbandry of the University of Wisconsin were used to supply the oöcysts and tissues for this investigation. Those used to produce oöcysts weighed approximately 700 grams, while those used in studies on the respiration of the coccidia within the epithelial cells of the cecal pouches weighed approximately 300 grams. All chickens were inoculated with approximately 50,000 sporulated oöcysts and were placed in sterile wire cages with outside feed and water containers at the time of infection.

Preparation of unsporulated oöcysts. On the 9th day after infection the birds were sacrificed and the cecal pouches removed. The cores and scrapings of the cecal pouches containing the oöcysts were placed in the Waring blender with sufficient water to just cover the blades. From 3 to 5 minutes were found to be sufficient to separate the oöcysts from the cecal material.

At the end of the mixing period a sufficient amount of water was added to increase the volume about 3 times. The resulting mixture was placed in a large separatory funnel and allowed to stand for 30 minutes. The sediment was then transferred to 50-cc centrifuge tubes.

The method of concentrating the oöcysts and washing them free of bacteria was a modification of that used by Lane (1922) for concentrating helminth eggs and by McCoy (1929) for the sterilization of hookworm eggs. The sediment was washed clear of much of the extraneous material by repeatedly centrifuging with top water at a speed of about 1000 revolutions per minute. When the supernatant fluid became clear, saturated sodium chloride solution was added to the sediment and the

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tubes again centrifuged. The resulting surface film containing the oöcysts was transferred to another centrifuge tube by means of a piece of glass tubing or the open end of a small vial. The transferred oöcysts were centrifuged again with sterile distilled water to free them of the concentrated salt solution.

Bacteriological counts of the oöcyst suspension after repeated washing and flotation with salt solution revealed only a small number of bacteria. To free the suspension of the few remaining bacteria, 5 per cent antiformin made up in 10 per cent formalin was added and the mixture allowed to stand for 5 minutes. The oöcysts were again concentrated by centrifuging and the antiformin-formalin solution poured off. To test the sterility of the suspension, 0.1 cc was inoculated into tubes of nutrient agar, which were incubated at 37° C for 24 hours. Only the respiratory determinations on the bacteria-free oöcyst suspensions are included in this report. The time required to prepare the oöcysts and the respiratory apparatus for the initial reading was approximately 4 hours.

The preparation of sporulated oöcysts.—The oöcysts to be sporulated were freed from the host tissue by means of the *Waring blender* as described above, and were then placed in a thin layer in Petri dishes and diluted about 5 times with 2.5 per cent potassium dichromate. The Petri dishes were left partially open to maintain adequate oxygen in the solution.

The procedure followed in preparing the sporulated oöcysts for respiratory determinations was much the same as that described for unsporulated oöcysts. The sporulated oöcyst suspension was passed through a 100-mesh copper sieve to remove larger particles. The filtrate was centrifuged several times in water to remove the potassium dichromate solution and most of the extraneous material. Saturated sodium chloride solution was used for oöcyst flotation and 5 per cent antiformin made up in 10 per cent formalin solution was used as described above to kill the bacteria.

Method of counting the oöcysts.—To count the oöcysts, 0.1 cc of oöcyst suspension was diluted with 0.9 cc of water. After thorough mixing a drop of the suspension was placed on a Spencer Bright-Line hemacytometer. The öocysts in 5 of the millimeter squares were counted and the number multiplied by 2 to get the number for 1 cubic millimeter. The final values were all expressed in cubic centimeters.

The respiratory apparatus, method of calculation, and solutions.—The Warburg apparatus was used throughout this investigation for respiratory determinations. The method of calculating the data was according to that of Dixon (1934). To calculate the amount of oxygen absorbed in the respiratory flasks from the manometer readings, it was necessary to know the manometric constant for each of the flasks except that used as the thermobarometric flask. These constants were calculated by substituting the proper values in the following equation and simplifying:

$$Ko_2 = \frac{V_g \frac{273}{T} \text{ plus } V_f a}{P_o}$$

where V_g is the volume of gas between the surface of the liquid of the flask and the 150-millimeter mark on the manometer, V_f is the volume of the liquid within the flask, T is the absolute temperature of the water bath, and P_o is the pressure of the Brodie solution of the manometers. Once the constant (Ko_2) for a flask

at a given temperature was known, the rate of oxygen consumption could be readily calculated by use of the following formulas:

$$\text{for oöcyst suspensions: } Qo_2 = \frac{hKo_2 \times 10^6}{\text{number of oöcysts}}$$

$$\text{for tissue respiration: } Qo_2 = \frac{hKo_2}{\text{dry weight of tissue}}$$

where h is the reading of the manometer in cubic millimeters.

Phosphate buffers were used as the substrate in most of the experiments and the different pH intervals prepared by mixing in different proportions, 0.1 M potassium monobasic phosphate and 0.1 M potassium dibasic phosphate. For pH ranges below 4.5, KCl–HCl buffers and phthalate buffers were prepared according to the method described by Clark in his book on the determination of hydrogen-ion concentration.

PRESENTATION OF DATA

The respiration of cecal pouch tissue parasitized by E. tenella.—To determine the respiratory rate of normal tissues and that of tissues parasitized by *Eimeria,*

TABLE 1.—*Showing the difference between the O_2 consumption of normal and parasitized cecal pouch tissue.* (Qo_2 = *cmm O_2 per mgm of tissue per hour*)

Experiment No.	Normal cecal pouch tissue			Parasitized cecal pouch tissue		
	Flask No.	Qo_2	Wt. of tissue in mgm	Flask No.	Qo_2	Wt. of tissue in mgm
1	2	7.5	4.8	4	10.7	3.4
	3	5.8	5.4	6	6.6	4.4
2	1	4.8	3.0	4	7.8	4.0
	2	3.7	4.0	6	4.3	3.9
				7	6.0	3.1
3	1	3.2	6.0	4	10.4	6.7
	2	5.5	4.4	6	7.0	6.3
4	1	3.3	6.3	3	7.9	5.2
	2	5.5	11.0	6	8.7	5.0
				7	7.4	6.0
5	1	4.8	4.5	4	7.9	4.0
	2	3.7	3.0	6	4.3	3.9
				7	6.0	3.1
Mean		4.7 ± .08			7.3 ± .11	

small, relatively uniform samples were removed from the cecal pouches of the chickens by means of a tonsil punch, 4 millimeters in diameter. The tissue samples were removed as quickly as possible (a few seconds after the chickens were killed), washed in sterile saline, then in a 1 to 1 mixture of a phosphate buffer (pH 7.3) and 1–500 solution of sulfanilamide. Two cc of the same solution were placed in the respiratory flasks to serve as substrate. The temperature of the flasks was maintained at 38° C and readings of the manometers were taken at 30-minute intervals over a period of 2 hours. At the termination of the experiment the tissues were removed to a weighing bottle and reduced to a constant weight in a drying oven. The dry weights were used to calculate the oxygen consumption and all rates were expressed in terms of oxygen consumed per milligram of the dry tissue per hour. Two Warburg flasks containing normal tissues and three containing parasitized tissue were run simultaneously.

The results of experiments to determine the respiratory rate of normal and parasitized tissue are shown in Table 1. The mean Qo_2 for the 10 determinations on

normal cecal pouch tissue was 4.7, and that for the 13 determinations on parasitized tissue was 7.3. The data on the oxygen consumption of both types of tissues were analyzed statistically and found to be significantly different. It must be concluded, therefore, that the parasites had a significant effect on the oxygen consumption of the cecal pouch tissue.

Normal respiration of oöcysts.—Normal respiration in this case is that of oöcysts placed under conditions known to be satisfactory for practical laboratory work. When oöcysts are kept at room temperature or slightly above (30° C) and at a pH of approximately 7.3, they sporulate rapidly and uniformly, and may then remain viable for over two years. The temperature of 30° C and pH 7.3 were taken as the normal. The number of oöcysts in each Warburg flask varied among the different experiments and ranged from 3,000,000 to 12,000,000 per cc. The number of oöcysts per flask was the same in any one experiment and the calculations were all reduced to the amount of oxygen per million oöcysts. Determinations were made for varying periods with readings taken at half-hour intervals.

TABLE 2.—*The rate of oxygen consumption of unsporulated oöcysts during the first twelve hours.* (Qo_2 = *cmm* O_2 *per million oöcysts per hour*)

Exp. No.	Flask No.	1st hr.	2nd hr.	3rd hr.	4th hr.	5th hr.	6th hr.	7th hr.	8th hr.	9th hr.	10th hr.	11th hr.	12th hr.	Number of oöcysts
2	1	12.2	10.0	13.0	11.2	9.0	12.0	11.0	8.0	10.0	14.0	20 2	26.0	3.560,000
4	3	20.8	12.7	17.3	15.0	17.3	17.3	15.0	16.1	13.8	23.1	27.7	39.3	3,000,000
4	6	14.5	15.3	16.6	15.0	16.6	15.8	29.8	8.3	14.0	15.3	35.3	38.9	4.000,000
28	1	8.0	6.9	10.2	7.0	7.1	7.2	6.9	6.8	8.0	10.0	18.9	31.0	2,000,000
30	1	18.9	9.4	18.9	14.2	17.8	16.6	14.2	14.2	14.2	13.0	27.3	37.9	2,000,000
30	2	19.2	13.2	18.0	18.8	16.2	18.0	15.6	14.4	14.4	13.2	26.4	38.4	2,000,000
Average		15.6	11.2	16.6	12.7	14.0	14.4	15.3	11.3	12.4	14.7	26.0	35.2	

Data on the unsporulated oöcysts are presented in Table 2 and show that little change in Qo_2 occurred during the first 10 hours. There was however, a great change following this period. The Qo_2 for the first 10 hours ranged from 11.3 to 15.6. During the 11th hour the Qo_2 increased to 26.0 and on the 12th hour was 35.2. This increase is in accord with the findings of George Ott (unpublished data) who found that there was little visible change in the protoplasm of the oöcysts during the first 12 hours, but a marked change during the second 12-hour period. Circumstances beyond control prevented the completion of the Qo_2 determinations between the 12- and 48-hour period. By the time the oöcysts were 48 hours old, the Qo_2 had decreased to about 0.4.

There was considerable variation in the Qo_2 of the various batches of oöcysts but when the data were examined statistically the variation was not significant.

The determinations on the normal rate of respiration of sporulated oöcysts were performed under conditions similar to those for unsporulated oöcysts. The temperature of the water bath of the respirometer was 30° C, the pH of the substrate was 7.3, and the length of the determinations was 3 hours. The concentrations of the oöcyst suspensions used were from 5 to 12 millions. The results of these determinations are given in Table 3. An inspection of the Qo_2 values shown in this table will reveal the marked difference between unsporulatd and sporulated oöcysts as to rates of respiration.

The number of oöcysts used in the 23 determinations on sporulated oöcysts may

TABLE 3.—*The rate of O_2 consumption of sporulated oöcysts over a period of three hours. (Qo_2 = cmm O_2 per million oöcysts per hour)*

Exp. No.	Flask No.	Qo_2	No. of oöcysts
21	6	1.00	12,420,000
20	3	0.60	12,300,000
18	1	0.61	11,500,000
14	2	0.23	10,000,000
14a	3	0.16	10,000,000
14b	4	0.23	10,000,000
14c	6	0.33	10,000,000
14d	1	0.20	10,000,000
13	1	0.30	7,920,000
13a	2	0.50	7,920,000
13b	3	0.60	7,920,000
13c	4	0.40	7,920,000
13d	6	0.40	7,920,000
13e	7	0.50	7,920,000
11	1	1.30	7,330,000
10	1	0.38	7,000,000
10a	2	0.56	7,000,000
15	1	0.33	7,000,000
15a	2	0.36	7,000,000
15b	3	0.46	7,000,000
12	1	0.90	6,000,000
16	1	0.26	5,670,000
16a	3	0.46	5,670,000

be considered in 4 classes: (1) 6 to 7 millions, (2) 8 millions, (3) 10 millions, (4) 11 to 12 millions. The average Qo_2 for the 4 classes in their order were 0.5, 0.4, 0.2, and 0.7. These values show on inspection a decrease in oxygen uptake as the number of oöcysts used increased from 6 to 10 million, but above 10 million the rate increased over all other values. Statistical analysis was made of values in two of the number classes to determine the significance of the difference between the mean values of the groups. The two classes selected were the 8 million (Exp. 13) and the 10 million class (Exp. 14). The mean rate for the 10 million class was 0.3 mm^3 per million oöcysts per hour, and the standard deviation was 0.15 With the 8 million the mean was 0.8 and the standard deviation 0.11. When these values were substituted in the proper statistical formulas, no significant difference was revealed between the 2 means. The oxygen uptake of the sporulated oöcysts is so small, and the variations between rates taken at different times is so great that it is probable that a much larger number of determinations will have to be made before any distinct trend in uptake due to numbers can be detected. Some of the possible reasons for the variations seen between runs will be discussed later.

The effect of pH on oxygen consumption of oöcysts.—Six experiments were conducted to determine the effect of a given pH range on the oxygen consumption of sporulated oöcysts. Each of the experiments consisted of 3 flasks with 2 cc of phosphate buffer and 1 cc of an oöcyst suspension. The pH intervals used throughout the series were 4.7, 7.3, and 8.8. The method of preparation of buffers at the various pH intervals was according to that described under the section on

TABLE 4.—*The effect of pH on the Qo_2 of sporulated oöcysts of* Eimeria tenella. *(Qo_2 = cmm of O_2 per million oöcysts per hour)*

Exp. No.	No. of hours observation	Rate of O_2 consumption in phosphate buffer at different pH intervals			No. of oöcysts used
		pH 4.7	pH 7.4	pH 8.8	
17	6	0.24	0.22	0.22	7,000,000
18	6	0.72	0.64	0.71	11,500,000
19	6	0.82	0.80	0.93	16,000,000
20	6	0.22	0.20	0.20	12,300,000
21	6	0.96	0.92	0.90	12,420,000
22	6	0.43	0.44	0.40	18,000,000

materials and methods. Each of the experiments ran for 6 hours and the Qo_2 values, which are given in Table 4 represent averages of the rates of oxygen consumption for the total length of each experimental run.

Because of the large amount of variation in rates of oxygen uptake between determinations made at different times and on different oöcyst suspensions, any conclusions drawn as to the effect of pH on the rates of oxygen consumption must be based on comparisons of the values within each experiment. Some of the differences may be due to differences in the number of oöcysts in the various experiments and some may be due to some physiological differences in the oöcysts themselves at the time of the experiments. In spite of these differences, the outstanding fact is that the pH values within the range considered had no significant effect on the rate of respiration of sporulated oöcysts.

Since sporulated oöcysts exhibited such a high resistance to the effects of pH, a characteristic resistance which these organisms show toward other chemical factors, it was surmised that a different type of response might be produced with unsporulated oöcysts. To test this hypothesis an experiment was performed with

TABLE 5.—*The effect of pH on the respiration of unsporulated oöcysts*

Flask No.	Type of substrate	pH of substrate	Rate of O_2 consumption in cmm/10^6/hr.	Number of oöcysts
1	KCl–HCl	1.3	16.0	2,000,000
2	Phthalate	2.4	16.4	2,000,000
3	PO_4–buffer	4.7	15.6	2,000,000
4	PO_4–buffer	7.3	15.3	2,000,000
5	PO_4–buffer	8.8	15.6	2,000,000

unsporulated oöcysts in substrates at 5 different pH intervals. The substrates used were KCl–HCl buffer at pH 1.3, phthalate buffer at pH 2.4, and phosphate buffers at pH 4.7, 7.3, and 8.8. The data (Table 5) reveal that differences in rates are too small to be attributed to differences in pH of the substrates. On the basis of all experiments presented it must be concluded that pH of the substrate within the limits tested has no effect on the respiration of oöcysts which can be detected by the methods used in this investigation.

DISCUSSION

Respiration of normal and parasitized tissue.—When the epithelium of the cecal pouches of chickens infected with *Eimeria tenella* is examined it is not surprising to find a significant increase in the respiratory rate of the parasitized tissue. The parasitized cells are greatly enlarged; the submucosa is packed in many areas with displaced epithelial cells and the small capillaries normally present have become greatly distended with blood. The general picture is that of an inflammatory reaction. While it is not difficult to imagine an increase in metabolism of the parasitized tissue, it is not easy to explain just how much of the increase is due directly to the parasites themselves and to the increase due to the pathology of the tissues. It was not the purpose of this study to determine the cause of the increase but rather to measure the change in the metabolic rate of the parasitized cells if a change did occur.

Since the tissues studied were not completely freed of bacteria, the effect of these organisms on the results of the experiments must be considered. The possibility

that the bacteria were responsible for the difference in the metabolic rate of the parasitized and normal tissues seems unlikely. The tissues were prepared in exactly the same manner, and George Ott (unpublished data) found no significant change in the number of types of bacteria present during the development of coccidiosis.

Respiration of sporulated and of unsporulated oöcysts.—In the unsporulated oöcysts there is a rounded mass of undifferentiated protoplasm. During the first 24 hours following discharge of the oöcysts into a favorable external environment, the oöcyst undergoes a division into 4 spores each containing 2 sporozoites. Within 48 hours practically all of the visible oöcysts have undergone complete development and have entered the resting stage with a resulting greatly reduced metabolic rate.

When the data on the metabolism of the 2 stages of oöcysts are examined the difference in Qo_2 is obvious. It is also evident that there existed a wide variation in the metabolic rate of oöcysts produced by different groups of chickens. No attempt will be made to explain this variation. That point alone may require years of concentrated study to solve. There are, however, several factors which can be mentioned that may throw some light on the subject.

The oöcysts are the product of the second generation merozoites which are released on the 5th day following infection. With this release there is a sudden and great decrease in the number of erythrocytes due to hemorrhage (Herrick, Ott, and Holmes, 1936). There is also a drop in hemoglobin, a great decrease in non-protein and total nitrogen, and concomitant with these changes, there is a great increase in blood sugar (Pratt, 1940, and Waxler, 1941) and blood chlorides (Waxler, 1941). It is during the 6th and 7th day following infection that the temperature may drop several degrees and the greatest mortality occurs. The mortality from coccidiosis may reach 100 per cent but that is unusual even from heavy artificial infections. In nearly every infection some chickens recover. It is only those that pass through the various pathological changes and recover that ever produce oöcysts. It is conceivable, therefore, that any one of the factors mentioned may affect the metabolism of the resulting oöcysts. It has been discovered by S. A. Edgar (to be published soon) that late on the 6th day, the macrogametocytes store relatively large quantities of glycogen. Undoubtedly other materials are stored at this time for use in the later sporulation process. It has been shown that canaries infected with malaria are more severely affected if fed sugar (Hegner and MacDougall, 1926), and rats infected with some pathogenic trypanosomes survive longer if starved or injected with insulin than if given normal treatment and feed. It is also reasonable to think that chickens which exhibit a greater resistance to coccidiosis may greatly affect the physiology of the oöcysts produced. It is conceivable that the amount of blood sugar in the blood of the chicken, the nitrogen level, or the amount of hemoglobin in the blood could affect the amount or quantity of materials stored by the gametocyte and thus affect their metabolic rate during subsequent development.

It is soon learned by anyone working with coccidian oöcysts that certain batches remain viable for long periods, two years or more, while others lose their infectivity within a few months. Whatever factor or factors affect the viability of the oöcysts may also cause the variation in metabolism observed among oöcysts secured from different chickens. Such explanation must await future work.

The effect of pH on respiration.—Cases in which the respiratory rates have been

shown to vary in substrates of different pH have been reported for several phyla. Hiestand and Hale (1938) found the respiratory rate of fresh-water molluscs to decrease when the pH was lowered. Hiestand (1940) found the respiratory rate of *Thyone* (Holothurian) to increase as the pH increased from 5.4 to 8.8. Von Brand (1943) found that oxygen consumption of *Eustrongylus* (nematode) remained practically unchanged in the pH range from 3.4 to 8.3. An increase in oxygen consumption was, however, observed in the two pH ranges 1.1 to 2.0 and 9.0 to 10.7. Maier and Coggeshall (1941) found that the respiratory rate of *Plasmodium knowlesi* remained constant between pH 7.0 and 8.0 but declined with further increase in pH up to 9.0.

In the present study no significant change in the rate of respiration of unsporulated oöcysts within the pH range of 1.2 to 8.8 and of sporulated oöcysts within the pH range of 4.5 to 8.8 was observed. These results are in accord with the findings of Horton-Smith and others (1940) who found that the unsporulated oöcysts of *E. tenella* could withstand exposure to sodium hydroxide (pH 11.2) for 2 hours and concluded that the effects of ammonia on *E. tenella* were due to toxic action of the drug rather than to effect of the change in hydrogen-ion concentration.

SUMMARY

1. The oxygen consumption of tissue parasitized with *E. tenella* was significantly higher than that of normal tissue.

2. The respiration of unsporulated oöcysts is from 10 to 20 times greater than that of sporulated oöcysts.

3. The respiration of oöcysts remained fairly constant within the pH range of 1.2 to 8.8.

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